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Callus cultures of *Thymus vulgaris* and *Trifolium pratense* as a source of geroprotectors

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Abstract.

Introduction. Geroprotectors are biologically active substances that inhibit the aging process. Many plant species are natural geroprotectors. For instance, *Thymus vulgaris* and *Trifolium pratense* are callus cultures with strong geroprotective properties.

Study objects and methods. The present research featured *T. vulgaris* and *T. pratense* grown *in vitro* on various nutrient media. Their extracts were obtained by aqueous-alcoholic extraction using the following parameters: water-ethanol solvent $S_e = 30, 50, \text{ and } 70 \%$; temperature $T_e = 30, 50, \text{ and } 70^\circ\text{C}$; time $\tau_e = 2, 4, \text{ and } 6 \text{ h}$. The quantitative and qualitative analysis was based on high-performance liquid mass spectrometry, gas mass spectrometry, and thin-layer chromatography.

Results and discussion. The optimal extraction parameters for *T. vulgaris* were $\tau_e = 4 \text{ h}$, $T_e = 50^\circ\text{C}$, $S_e = 70 \%$, for *T. pratense* – $\tau_e = 6 \text{ h}$, $T_e = 70^\circ\text{C}$, $S_e = 70 \%$. The chromatography detected flavonoids, phenylpropanoids, simple phenols, higher fatty acids, mono- and sesquiterpenes, and aliphatic hydrocarbons. *T. vulgaris* appeared to have the highest content of thymol ($23.580 \pm 1.170 \text{ mg/mL}$); its thymol, apigenin, gallic, chlorogenic, and caffeic components demonstrated geroprotective properties. The extract of *T. pratense* possessed the highest rutin content ($10.05 \pm 0.35 \text{ mg/mL}$), and it owed its geroprotective characteristics to rutin, chlorogenic and *p*-coumaric acids.

Conclusion. The callus cultures of *T. vulgaris* and *T. pratense* proved to be promising sources of geroprotective biologically active substances.

Keywords. Extraction, geroprotectors, *Thymus vulgaris*, *Trifolium pratense*, biologically active substances

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Каллусные культуры *Thymus vulgaris* и *Trifolium pratense* – перспективные источники активных веществ геропротекторного действия

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Аннотация.

Введение. Естественными источниками биологических активных веществ (БАВ) являются различные виды растений, обладающие геропротекторными свойствами, замедляющие процессы старения. *Thymus vulgaris* и *Trifolium pratense* обладают высоким содержанием БАВ. Данная работа посвящена исследованию экстрактов каллусных культур *T. vulgaris* и *T. pratense* на наличие веществ геропротекторной направленности.

Объекты и методы исследования. Для получения экстрактов использовались каллусные культуры *T. vulgaris* и *T. pratense*, выращенные в условиях *in vitro* на питательных средах различного состава. Для извлечения веществ выбран метод жидкостной водно-спиртовой экстракции. В качестве растворителя использовалась водно-этанольная смесь ($C_3 = 30, 50, 70 \%$). Также варьировали следующие параметры экстракции: температуру $T_3 = 30, 50, 70 \text{ }^\circ\text{C}$, время $\tau_3 = 2, 4, 6 \text{ ч}$. Количественный и качественный анализ экстрактов каллусных культур *T. vulgaris* и *T. pratense* исследовали с применением высокоэффективной жидкостной, газовой с масс-спектрометрией и тонкослойной хроматографии.

Результаты и их обсуждение. Для получения экстрактов каллусных культур рекомендованы следующие рабочие параметры: для *T. vulgaris* – $\tau_3 = 4 \text{ ч}$, $T_3 = 50 \text{ }^\circ\text{C}$, $C_3 = 70 \%$, для *T. pratense* – $\tau_3 = 6 \text{ ч}$, $T_3 = 70 \text{ }^\circ\text{C}$, $C_3 = 70 \%$. По результатам хроматографического исследования экстрактов установлено наличие флавоноидов, фенилпропаноидов, простых фенолов, высших жирных кислот, моно- и сескитерпенов и алифатических углеводов. В *T. vulgaris* наибольшее содержание тимола ($23,580 \pm 1,170 \text{ мг/мл}$), а в *T. pratense* наибольшее содержание рутина ($10,05 \pm 0,35 \text{ мг/мл}$). Из найденных веществ в экстракте *T. vulgaris* геропротекторной активностью обладают тимол, апигенин, галловая, хлорогеновая и кофейная кислоты; а в *T. pratense* рутин, хлорогеновая и *n*-кумаровая кислоты.

Выводы. Каллусные культуры *T. vulgaris* и *T. pratense* являются источниками БАВ геропротекторной направленности.

Ключевые слова. Экстракция, геропротекторы, *Thymus vulgaris*, *Trifolium pratense*, биологические активные вещества

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Introduction

Environment directly affects human health [1]. Urban environment is a reliable source of various noxious factors [2, 3]. One's health status depends on how well the body has adapted to the environment, while one's functional capabilities are based on the physiological profile, age, and character [4]. Any disease comes from this or that violation of adaptive mechanisms, i.e. when the body fails to resist unfavorable environmental conditions, e.g. air pollution, water contamination, vibration, radiation, noise, electromagnetic, etc. [5–7].

Plant materials can resolve various health issues and inhibit early aging [8]. Plant extracts are the sources of beneficial biological active compounds that can prevent aging, increase life expectancy, improve physical, mental, emotional, and social state, etc. [9–14].

Biologically active compounds of plant origin can be divided into several chemical groups. Glycosides include cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins, and anthraquinone glycosides. Phenolic compounds involve phenolic and hydroxycinnamic acids, stilbenes, flavonoids, and anthocyanins. Tannins are divided into condensed tannins, e.g. large polymers of flavonoids, and hydrolysable tannins, which consist of a monosaccharide nucleus with several catechin derivatives attached. The list of biologically active

compounds also includes mono-, di- and sesquiterpenoids, phenylpropanoids, lignans, resins, alkaloids, furocoumarins and naphthodianthrons, proteins, and peptides [15, 16].

Secondary plant metabolites are potential geroprotectors. Their list includes flavonoids, polyphenols, glycosides, tannins, and vitamins. Other compounds that are believed to possess geroprotective properties are rutin, ononin, genistein, rapamycin, carvacrol, resveratrol, apigenin, metformin, terpenene, thymol, gallic acid, isoquercetin, oleanolic acid, *p*-coumaric acid, and various senolytics. Experiments on mice confirmed that these compounds improve cognitive, neuromuscular, metabolic, and immune systems, inhibit cataracts, sarcopenia, osteoarthritis, osteoporosis, atherosclerosis, Alzheimer's disease, and various age-related tissue dysfunctions [17].

Extraction is the main technological process that produces biologically active compounds from plant raw materials [18]. Extraction process includes three main stages: (1) interaction of plant material with the extractant, (2) destruction of plant cell components, and (3) transfer of solutes to the extractant [19, 20].

Extractants have to be able to penetrate cell walls and selectively dissolve biologically active compounds inside the cell. Therefore, a good extractant has to meet certain requirements, e.g. maximum solubility of active substances; selectivity; high penetrating power;

safety for the human body; volatility, and a low boiling point [21].

All extraction solvents are divided according to polarity. Polar extractants include water, glycerin, etc. They dissolve salts, alkaloids, glycosides, saponins, traglycosides, furocoumarins, organic acids, etc. Aqueous-alcoholic solutions have similar properties. Low-polarity extractants include ethanol, isopropyl, and butyl. They extract salts, alkaloids, flavones, carotenoids, essential oils, pigments, chlorophyll, glycosides, etc. Non-polar extractants include chloroform, hexane, benzene, etc. They extract flavones, essential oils, alkaloids, sapogenins, waxes, fats, etc. Ethanol and water are the most common solvents [22, 23]. As a rule, extraction and isolation of biologically active substances from natural sources follow a well-established procedure: exhaustive extraction (maceration, steam or hydrodistillation, pressing, decoction, infusion, percolation, and Soxhlet extraction); additional chemical processing to isolate the target compounds [13].

Plants are known to synthesize and accumulate secondary metabolites of various phytochemical groups [24, 25]. Callus, suspension, and root cultures are induced for analytical and quantitative comparative analyses of the secondary synthesis of metabolites between plant material and callus, suspension, and root extracts [22, 23].

Among all the medicinal wild plants of the Siberian Federal District, *Thymus vulgaris* and *Trifolium pratense* contain the most impressive amount of geroprotective biologically active substances, including such antioxidants as flavonoids, coumarins, etc. [26].

T. vulgaris has antiseptic, antimicrobial, and antioxidant properties [27]. Its extracts are used to treat dyspepsia and gastrointestinal disorders, cough, whooping cough, bronchitis, laryngitis, and tonsillitis, since it contains benzyl alcohol, rutin, apigenin, thymol, gallic acid, luteolin, etc. [28, 29]. *T. vulgaris* owes its high antimicrobial and antifungal activity to such phenolic compounds as thymol and carvacrol [26]. The yield of essential oil ranges from 0.3 to 6.3% [30]. The content of thymol in the essential oil can reach 60%, which is significantly higher than the content of carvacrol (up to 6%) [31]. The antiseptic effect of thymol is 30 times higher than that of phenol, while its toxic effect is 4 times lower [32]. Phenolic compounds of *T. vulgaris* can form oxygen free radicals [33].

T. pratense is used as animal feed. This melliferous plant is very popular in agriculture [34]. As a result, its biologically active substances have become focus of constant scientific attention. *T. pratense* contains some flavonoids, isoflavonoids, and phenolic compounds, e.g. quercetin, rutin, genistein, formononetin, etc. [32]. This plant is used as an antioxidant, antimicrobial and diuretic medicine, as well as a remedy against coronary and nephric edema [34–36].

The research objective was to perform a quantitative and qualitative analysis of callus extracts of *T. vulgaris* and *T. pratense* to evaluate their geroprotective prospects.

Study objects and methods

The research featured callus cultures obtained from seeds of *Thymus vulgaris* and *Trifolium pratense* grown *in vitro*. The seeds were washed in soapy water for 30 min and then washed in bidistilled water three times for 20 min. After that, they were treated with 70% ethanol for 1 min and washed three times in bidistilled water for 20 min. Finally, the seeds were washed with 5% sodium hypochlorite solution for 50 min and washed three times in bidistilled water for 20 min [37]. After sterilization, the seeds were planted on agar media. Table 1 shows the composition of the nutrient media.

The first seedlings of *T. pratense* appeared on week 1–2, and those of *T. vulgaris* – on week 4–5. The experiment featured sterile seedlings that were 2–5 weeks old. The explants were cut into pieces and planted in agar media. The first calli appeared during 14 days of cultivation. Further callus formation involved Murashige-Skoog (MS), Gamborg (B5), and Schenck Hildebrandt (SH) mineral bases with casein hydrolyzate (0.5 g/L), inositol (0.1 g/L), 3% sucrose or glucose, and 2% agar. The media varied in the composition of growth regulators, which included indoleacetic acid, 2,4-dichlorophenoxyacetic acid, kinetin, and 6-benzylaminopurine (Table 2). The explants were incubated for 25 days.

The primary callus was separated from the remains of the explants and transferred to a fresh nutrient medium to grow for 4–5 weeks.

The callus cultures were extracted by the standard method of liquid aqueous-alcoholic extraction using ethanol (State Standard 5962-2013. Rectified ethyl

Table 1. Nutrient media for seedlings of *Thymus vulgaris* and *Trifolium pratense*

Components	Per 1 l of distilled water	
	<i>Thymus vulgaris</i>	<i>Trifolium pratense</i>
B5 macrosalts (20×)*, mL	50.00	50.00
B5 microsals (20×)*, mL	10.00	10.00
Fe-EDTA, mL	5.00	5.00
Sucrose, g	30.00	30.00
Nicotinic acid, mg	1.00	0.50
Pyridoxine, mg	1.00	0.10
Thiamine, mg	10.00	0.10
Inositol, mg	100.00	100.00
6-benzylaminopurine, mg	–	1.00
Activated carbon, g	–	20.00
Agar, g	20.00	20.00
pH	5.4–6.0	5.4–6.0

* see T.A. Murashige [38].

Table 2. Mineral and hormone composition of the culture medium for the cultivation of callus cultures of *Thymus vulgaris* and *Trifolium pratense*

Components	Per 1 mL of distilled water					
	<i>Thymus vulgaris</i>			<i>Trifolium pratense</i>		
	Medium 1	Medium 2	Medium 3	Medium 1	Medium 2	Medium 3
MS macrosalt (20×)**, mL	50.00	50.00	50.00	50.0	50.00	50.00
MS macrosalt (20×)**, mL	1.00	1.00	1.00	10.0	10.00	10.00
Fe-EDTA, mL	5.00	5.00	5.00	5.00	5.00	5.00
Thiamine, mg	0.10	0.10	0.10	10.00	10.00	10.00
Pyridoxine, mg	0.50	0.50	0.50	1.00	1.00	1.00
Nicotinic acid, mg	0.50	0.50	0.50	1.00	1.00	1.00
Sucrose, g	30.00	30.00	30.00	30.00	30.00	30.00
Casein hydrolyzate, mg	500.00	500.00	500.00	–	–	–
Inositol, mg	100.00	100.00	100.00	100.00	100.00	100.00
Kinetin, mg	–	2.00	1.00	–	1.00	2.00
6-benzylaminopurine, mg	–	0.50	–	0.05	–	0.10
Indoleacetic acid, mg	–	–	2.00	1.00	–	2.00
Peoxyethanolic acid, mg	–	3.00	–	–	–	–
2,4-dichlorophenoxyacetic acid, mg	–	–	–	–	1.00	2.00
Agar, g	20.00	20.00	20.00	20.00	20.00	20.00
pH	5.4–6.0	5.4–6.0	5.4–6.0	5.4–6.0	5.4–6.0	5.4–6.0

** see O.L. Gamborg [39].

alcohol from edible raw material. Specifications) and distilled water (State Standard 6709-72. Distilled water. Specifications) as solvent [41]. The water was purified in a BS brand bidistiller (Labinvest, Russia).

The extraction parameters were as follows: extraction temperature – 30, 50, and 70°C; time – 2, 4, and 6 h, ethanol concentration – 30, 50, and 70%.

The callus cultures of *T. vulgaris* and *T. pratense* were dried and ground in an LZM-1M rotary mill (Olis, Russia). The extraction samples weighed 3.000 ± 0.001 g.

The volume of ethanol was 260 mL. The extraction was carried out in a water bath (Elmasonic S60H, Germany) with an ascending refrigerator at a given temperature, time, and ethanol concentration. The obtained extracts *T. vulgaris* and *T. pratense* were stored at room temperature in the dark.

The antioxidant activity of the extracts was determined to define the total biologically active substances. It was expressed as the content of the sum of biologically active substances of a reducing nature in terms of quercetin

Table 3. Antioxidant activity of *Thymus vulgaris* callus extracts

Temperature, °C	Volume fraction of ethanol, %		
	30	50	70
	Extraction time, 2 h		
30	0.2080 ± 0.0020	0.1250 ± 0.0012	0.1640 ± 0.0029
50	0.2250 ± 0.0023	0.1920 ± 0.0017	0.2240 ± 0.0030
70	0.1980 ± 0.0011	0.2180 ± 0.0030	0.2270 ± 0.0033
Extraction time, 4 h			
30	0.1576 ± 0.0016	0.2182 ± 0.0022	0.2048 ± 0.0036
50	0.1940 ± 0.0026	0.2134 ± 0.0030	0.2956 ± 0.0047
70	0.2687 ± 0.0031	0.2430 ± 0.0050	0.2873 ± 0.0050
Extraction time, 6 h			
30	0.2253 ± 0.0055	0.1939 ± 0.0030	0.2831 ± 0.0060
50	0.1811 ± 0.0046	0.2584 ± 0.0036	0.2752 ± 0.0049
70	0.1711 ± 0.0013	0.2388 ± 0.0050	0.2643 ± 0.0030

Table 4. Antioxidant activity of *Trifolium pratense* callus extracts

Temperature, °C	Volume fraction of ethanol, %		
	30	50	70
	Extraction time, 2 h		
30	0.1906 ± 0.0014	0.1464 ± 0.0010	0.1630 ± 0.0018
50	0.2122 ± 0.0030	0.2143 ± 0.0045	0.1703 ± 0.0040
70	0.1475 ± 0.0010	0.1847 ± 0.0032	0.1942 ± 0.0021
Extraction time, 4 h			
30	0.1741 ± 0.0030	0.1743 ± 0.0027	0.1460 ± 0.0040
50	0.1494 ± 0.0022	0.1852 ± 0.0016	0.1737 ± 0.0045
70	0.1046 ± 0.0010	0.1057 ± 0.0011	0.2392 ± 0.0056
Extraction time, 6 h			
30	0.1621 ± 0.0032	0.1329 ± 0.0010	0.1636 ± 0.0010
50	0.1419 ± 0.0018	0.1637 ± 0.0034	0.1813 ± 0.0021
70	0.1547 ± 0.0010	0.1386 ± 0.0026	0.1918 ± 0.0030

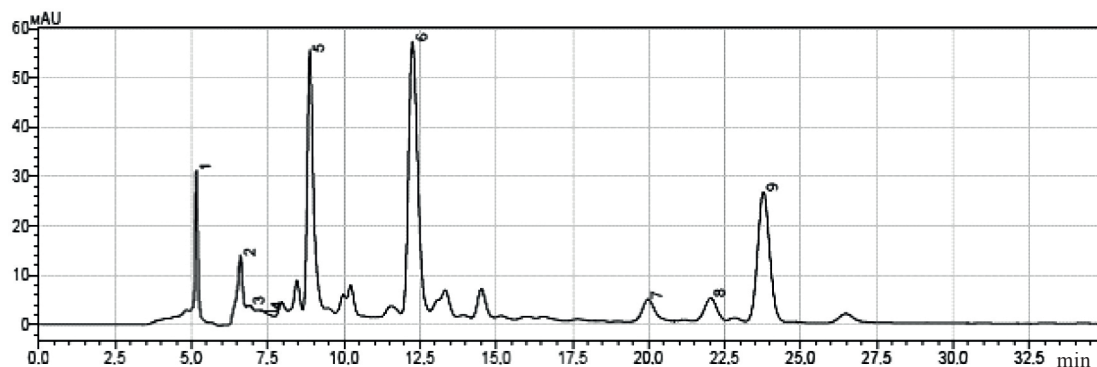


Figure 1. Chromatogram of the aqueous-alcoholic composition of *Thymus vulgaris* callus cultures:
1 – gallic acid; 2 – oleanolic acid; 3 – chloric acid; 4 – ursolic acid; 5 – apigenin-7-glucoside; 6 – caffeic acid;
7 – apigenin; 8 – carvacrol; 9 – thymolic acid

in 1 mL of the extract by the method developed by T.V. Maksimova [43].

The experiment involved a liquid chromatograph (Shimadzu LC-20 Prominence, Japan) with a Shimadzu SPD-20-MA diode array detector and a RID-10A refractometric detector, a chromatographic column Kromasil 5 μm C18, 250 \times 4.6 mm, a Guard Column Security Guard Gartridge (C18) Phenomenex (USA) with injection volume 20 μL . The column temperature was 30 $^{\circ}\text{C}$; the elution mode was isocratic; the mobile phase consisted of AcCN:isopropyl alcohol:H₂O–H₃PO₄ (20:5:75, pH 3.5).

Gas chromatography with mass spectrometry (GC-MS) and thin layer chromatography (TLC) were carried out at the same time as HPLC [44].

The analysis of biologically active substances involved Sorbfil PTS-AF-A TLC plates. The obtained extract was applied to the start line, dried, and placed in a chromatographic chamber filled with a mix of *n*-butanol, acetic acid, and water at a ratio of 60:15:25. After 10 min, a 25% solution of phosphoric-tungstic acid was added at 95 $^{\circ}\text{C}$. The densitometric analysis of the plate was performed using a Handycam HDR-CX405 densitometer with a Sony photofixation system (OOO IMID, Russia).

The *T. vulgaris* callus extracts underwent a GC-MS using a 30 m column with an inner diameter of 0.25 mm and helium as a carrier gas. The main parameters for GC-MS were as follows: carrier gas flow rate – 1.4 mL/min; interface temperature – 280 $^{\circ}\text{C}$; injector temperature – 240 $^{\circ}\text{C}$; column temperature – 100–270 $^{\circ}\text{C}$; volume of the injected sample – 3 μL . The sample was introduced without dividing the carrier gas flow.

Results and discussion

Nutrient medium 2 proved optimal for the callusogenesis of *Thymus vulgaris*, which included

the following growth hormones: kinetin – 2 mg, 6-benzylaminopurine – 0.5 mg, peoxyethanolic acid – 3 mg. When the callus culture of *Trifolium pratense* was cultivated on nutrient medium 2, which contained 1 mg of kinetin and 2,4-dichlorophenoxyacetic acid, the callus growth was slow. Nutrient medium 3 proved optimal for *T. pratense* callus culture: it contained the following growth hormones: kinetin – 2 mg, 6-benzylaminopurine – 0.1 mg, indoleacetic acid – 2 mg, and 2,4- dichlorophenoxyacetic acid – 2 mg.

Tables 3 and 4 show the total content of biologically active substances in terms of quercetin in 1 mL of the extract under different extraction conditions.

The obtained data demonstrated in Tables 3 and 4 made it possible to recommend the following optimal extraction parameters for *T. vulgaris*: τ_c – 4 h, T_c – 50 $^{\circ}\text{C}$, S_c – 70%; for *T. pratense*: τ_c – 6 h, T_c – 70 $^{\circ}\text{C}$, S_c – 70%.

After establishing the optimal extraction parameters, the next step was to analyze the qualitative and quantitative composition of biologically active substances in aqueous-alcoholic extracts.

Table 5. Component composition of *Thymus vulgaris* callus extract

Peak	Retention time, min	Component	Quantitative content, mg/mL
1	5.162	gallic acid	5.720 \pm 0.320
2	6.610	oleanolic acid	5.290 \pm 0.400
3	6.910	chloric acid	1.920 \pm 0.300
4	7.240	ursolic acid	2.200 \pm 0.200
5	8.860	apigenin-7-glucoside	11.460 \pm 0.760
6	12.240	caffeic acid	18.930 \pm 0.880
7	19.980	apigenin	3.180 \pm 0.320
8	22.045	carvacrol	8.170 \pm 0.490
9	23.930	thymolic acid	23.580 \pm 1.170

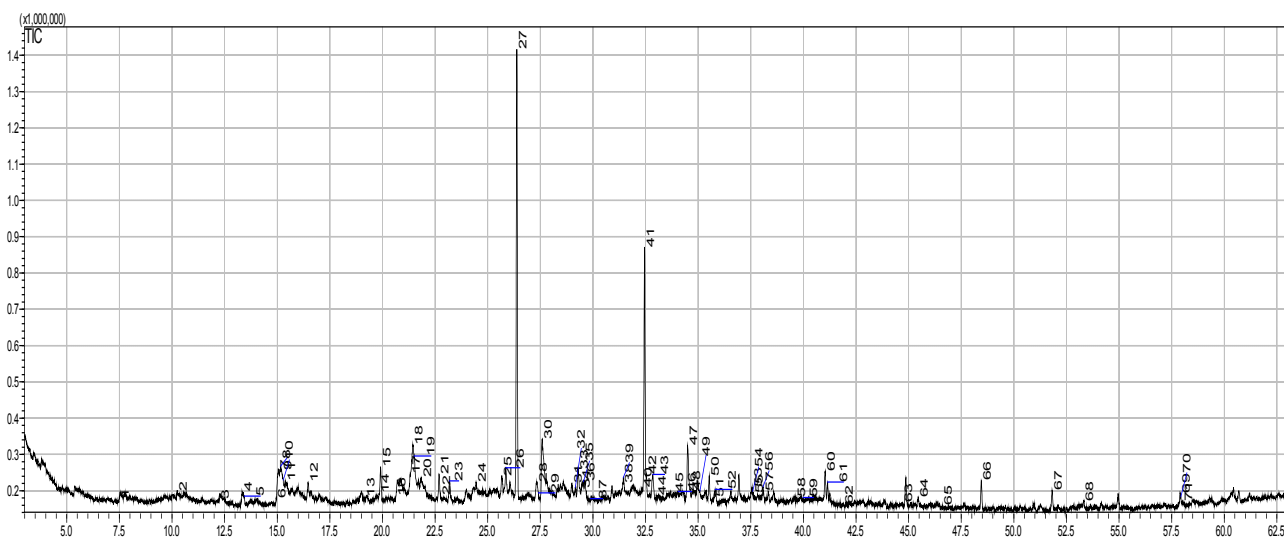
Figure 2. GC-MS chromatogram of *Thymus vulgaris* callus culture fraction

Figure 1 shows the results of HPLC analysis for *T. vulgaris* callus extract, while Table 5 demonstrates the results of the qualitative and quantitative analysis of biologically active substances.

The HPLC analysis (Fig. 1, Table 5) of *T. vulgaris* callus extracts revealed that the samples contained flavonoids, phenylpropanoids, and simple phenols.

Figure 2 illustrates the GC-MS analysis of *T. vulgaris* callus extract and displays other individual biologically active substances.

The GC-MS analysis of the composition of biologically active substances in the aqueous-alcoholic callus extracts of *T. vulgaris* revealed the following composition: mono- and sesquiterpenes – tricyclene, thuyenne, terpene, cubeben, verbenone, verbenol, eucalyptol, linalool, bourbonene, borneol, isborneol, caryophyllene, kadinen, farnesene, cadinol; higher fatty acids – 3-ketopentatriacontanic *n*-heneicosan; aliphatic hydrocarbons – heptadecane,

nonadecane, heneicosan, heptanol, octanol, 1-dodecanol, 3-octanone (Fig. 2).

The extracts of *T. pratense* callus culture underwent HPLC and TLC chromatography. Figure 3 demonstrates the HPLC chromatogram.

The HPLC analysis revealed biologically active substances that belong to phenolcarboxylic acids: *p*-coumaric acid, *p*-coumaroyl-3-quinic acid, and chlorogenic acid; flavones: rutin, isoquercitrin; isoflavones: daidzein, genistein. Table 6 demonstrates the qualitative and quantitative HPLC analysis of the sample.

The content of biologically active substances in *T. pratense* samples changed depending on the extraction method, which was the main peculiarity of this extract. After ultrasonic extraction, the amount of flavanoids was 2.13%, isoflavonoids – 4.42%; after heat maceration, the yield of flavonoids was 1.64%, isoflavonoids – 3.24% [40, 42]. Figure 4 shows the densitogram of the TLC analysis of the *Trifolium pratense* callus extract.

Table 6. Components of *Trifolium pratense* callus extract

Peak	Retention time, min	Component	Quantitative content, mg/mL
1	4.49	rutin	10.05 ± 0.35
2	6.15	chlorogenic acid	7.29 ± 0.42
3	7.25	<i>p</i> -coumaroylquinic acid	23.10 ± 0.74
4	9.03	<i>p</i> -coumaric acid	1.26 ± 0.10
5	9.70	isoquercitrin	1.55 ± 0.08
6	10.30	biochanin A	8.89 ± 0.35
7	13.50	ononin	2.51 ± 0.20
8	13.97	daidzein	1.36 ± 0.07
9	17.20	genistein	9.05 ± 0.13
10	47.01	melilotic acid	3.61 ± 0.19

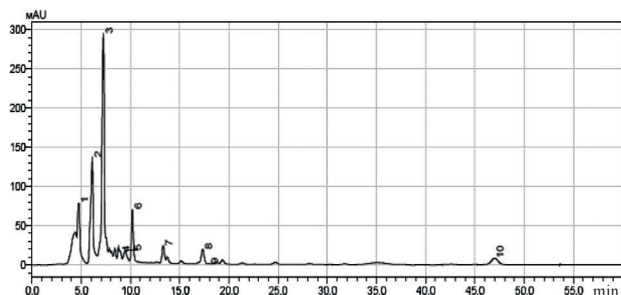


Figure 3. HPLC chromatogram of *Trifolium pratense* callus extract: 1 – rutin; 2 – chlorogenic acid; 3 – *p*-coumaroylquinic acid; 4 – *p*-coumaric acid; 5 – isoquercitrin; 6 – biochanin A; 7 – ononin; 8 – daidzein; 9 – genistein; 10 – melilotic acid

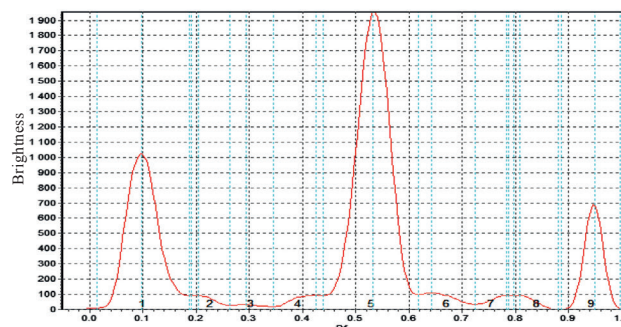


Figure 4. Densitogram of *Trifolium pratense* callus extract under TLC separation on a Sorbfil plate: Rf 0.1 – quercetin-3-O-rutinoside (rutin); Rf 0.53 – chlorogenic acid; Rf 0.95 – isoquercitrin

The TLC analysis showed that the *T. pratense* callus extracts contained such biologically active substances as quercetin-3-O-rutinoside (rutin), chlorogenic acid, and isoquercitrin.

Based on scientific publications and the chromatography performed, the *T. vulgaris* and *T. pratense* callus extracts proved to contain secondary metabolites with geroprotective properties. The callus culture of *T. vulgaris* contained gallic, oleanolic, chlorogenic, and caffeic acids, apigenin, carvacrol, thymol, terpene, verbenone, verbenol, isoborneol, caryophyllene, kadinene, farnesene, and cadinol [45–47]. The callus culture of *T. pratense* contained such geroprotectors as rutin, chlorogenic, and *p*-coumaric acid [48–51].

Conclusion

The present research featured the quantitative and qualitative content of biologically active substances in aqueous-alcoholic extracts of callus cultures of *Thymus vulgaris* and *Trifolium pratense*. The optimal extraction parameters for *T. vulgaris* callus culture were as follows: τ_e – 4 h, T_e – 50°C, S_e – 70%; for *T. pratense*: τ_e – 6 h, T_e – 70°C, S_e – 70%.

The qualitative and quantitative analyses of biologically active substances in the aqueous-alcoholic callus extracts of *T. vulgaris* and *T. pratense* were based on chromatography. The HPLC test revealed gallic, oleanolic, chlorogenic, and ursolic acids, apigenin-7-glucoside, caffeic acid, apigenin, carvacrol, and thymol in the *T. vulgaris* callus

extracts. The selected extraction parameters resulted in a high yield of thymol (23.580 ± 1.170 mg/mL). The GC-MS analysis revealed mono- and sesquiterpene (tricyclene, thujene, terpenene, cubebene, verbenone, verbenol, eucalyptol, linalool, bourbonene, borneol, isoborneol, caryophyllene, kadinene, farnesene, and cadinol), higher fatty 3-ketopentatriacontanic acid, aliphatic hydrocarbons (heptadecane, nonadecane, heneicosan, heptenol, octanol, 1-dodecanol, and 3-octanone).

As for the *T. pratense* callus extracts, the HPLC analysis revealed rutin, chlorogenic acid, *p*-coumaroyl-3-quinic acid, *p*-coumaric acid, isoquercitrin, biochanin A, ononin, daidzein, genistein, and melilotic acid. The selected extraction parameters produced a high yield of rutin (10.05 ± 0.35 mg/mL). According to the TLC chromatography, the *T. pratense* callus extracts contained rutin, chlorogenic acid, and isoquercitrin.

Therefore, the callus cultures of *T. vulgaris* and *T. pratense* proved to be sources of geroprotective biologically active substances.

Contribution

The authors are equally responsible for the information published in this article and any possible cases of plagiarism.

Conflict of interest

The authors declare no conflict of interests regarding the publication of this article.

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